OBSERVATIONS ON *ENTAMOEBA HISTOLYTICA*

II. LONGEVITY OF THE CYSTS *IN VITRO*, AND THEIR RESISTANCE TO HEAT AND TO VARIOUS DRUGS AND CHEMICALS

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(*Received for publication*, 26 *July*, 1926)

Hitherto we have had no absolutely satisfactory criterion which would permit us to decide whether *E. histolytica* cysts are alive or dead. The usually accepted method of determining the vitality of cysts is their behaviour in dilute eosin solution. This test, which was apparently first employed for the purpose of deciding whether *E. histolytica* cysts were viable by Kuenen and Swellengrebel (1913), is based on the hypothesis that dead cysts stain with weak solutions of eosin in water (1 in 1,000) and that living cysts do not. Although it was impossible to determine whether this hypothesis was wholly correct, it was generally accepted that those cysts which stained were certainly dead, but whether all those which did not stain were alive seemed to be a much more doubtful matter.

Another possible method for determining the vitality of *E. histolytica* cysts is to feed them to cats, but apart from the enormous sacrifice of animals which such a method would entail in an extensive investigation on the vitality of the cysts, and the time which it would occupy, the procedure is, as Kuenen and Swellengrebel have pointed out, so often attended by negative results, even when quite fresh material is used, as to be hardly suitable for this purpose. Quite recently Sellards and Theiler (1924) have shown that cats can be infected by intrarectal injection of *E. histolytica* cysts, but the use of this technique for determining the vitality of cysts is open to the same objections.

In a previous paper (1926) we have described a method of obtaining cultures of *E. histolytica* from cysts. This method, which has in our hands proved invariably successful, seems admirably adapted for testing the vitality of *E. histolytica* cysts, and in the present work we have employed it for determining their vitality in faeces kept *in vitro* for varying lengths of time, and under various conditions, and for examining the effect on the cysts of heat, and of various drugs and chemicals.

LONGEVITY OF *E. HISTOLYTICA* CYSTS *IN VITRO*.

This is, from the public health point of view, a subject of fundamental importance, but owing to the hitherto inadequate and inaccurate methods available for deciding whether *E. histolytica* cysts are viable, it is not surprising that the data obtained are somewhat scanty and highly conflicting.

Kuenen and Swellengrebel kept infected faeces at laboratory (27 to 30° C.) and at incubator (37° C.) temperatures, and found that after three days all the cysts had disappeared from the specimen kept at 37° C. ; in that kept at 27 to 30° C. all the cysts were alive after three days, but about half of them were dead by the fourth day, and all by the ninth day. The cysts from another portion of the same faeces were separated, so far as possible, from faecal material, by washing and centrifuging, and allowed to stand at 27 to 30° C. in water ; under these conditions practically all the cysts were found to be alive on the ninth day : subsequently enormous bacterial growth occurred, and this is stated quickly to have destroyed the cysts, so that hardly any living individuals were to be found by the thirteenth day ; nevertheless, a few are stated to have been still alive after 29 days. These conclusions were based on the eosin criterion.

Penfold, Woodcock, and Drew (1916), who judged of the viability of the cysts by their power to excyst in the presence of *liquor pancreaticus*, state that they were able to ' keep cysts in a very slowly running current of water for 15 days and to ascertain that certainly some were alive at the end of this period,' and they infer ' that water which has been contaminated with cyst-containing faeces may remain a source of infection for a considerable period.' Wenyon and O'Connor (1917) employing the eosin test for viability

write, that ' cysts will survive for over 30 days in water,' and that ' apparently the cysts survive best if there is considerable dilution of faeces with water, so that intense bacterial or fungoid overgrowth does not take place.'

Dobell (1919) writes as follows :—' The cysts of *E. histolytica* will survive for several weeks outside the body of man, if they are kept moist and cool. They will live in damp faeces or in water without showing any conspicuous change save the loss of their chromatoid bodies. As a rule, if the cysts are kept under observation, it will be found that some of them remain alive much longer than the others. In water or faeces some will usually be found dead at the end of a week, many more after the lapse of a fortnight, and after this period only isolated survivors will be discoverable. The longest time of survival which I have observed is five weeks (cysts kept in water), but as a rule they will not live so long. Desiccation kills them immediately, and they degenerate much more rapidly at a high than at a low temperature. At body temperature they generally die within a few days at most. Degeneration of the cysts is readily recognizable. The nuclei first become unnaturally distinct in the fresh cysts—owing to the coagulation which occurs on the death of the protoplasm—and then break up. As the cysts die they also become permeable to aqueous solutions of various stains (eosin, etc.). The cytoplasm becomes vacuolated, and finally disintegrates.'

Boeck (1921b), accepting the eosin-solution supplemented by morphological observations as the criterion of viability, states that when ' immersed in distilled water, contained in bottles and kept at a temperature of 12° to 22° C., cysts of *E. histolytica* were found viable at the end of 153 days ' ; and ' in eosin-stained wet preparations, sealed with vaseline, cysts of *E. histolytica* were viable at the end of 211 days.'

Summarising these records we find some indication that cysts live longer in water than in faeces, and that low temperatures are more favourable for longevity than are higher, or body temperatures. The actual period for which, under favourable conditions, the cysts will remain viable is stated to be very different by the various investigators, e.g., Kuenen and Swellengrebel found a few alive after 29 days, Wenyon and O'Connor state they will survive for over 30 days, Dobell found some alive up to five weeks, and Boeck states

that they may live for such lengthy periods as 153 and 211 days.

In re-investigating the subject we decided to ascertain the longevity of *E. histolytica* cysts, not only in the original faecal mass kept at 0° C. and at laboratory temperature (16 to 20° C.), but also in water suspensions—prepared by both of the methods described in our previous paper (1926)—kept at similar temperatures. In order to prevent bacterial overgrowth the suspensions were washed every three or four days by centrifuging, removing the supernatant fluid, and replacing it by fresh water or saline. The suspensions were tested for the presence of live cysts by sowing periodically five or six drops of the centrifuged deposit on L.E.S. medium, and examining the culture for vegetative *Entamoebae* in the manner previously described, after incubation at 37° C. for 16 to 20 hours. The cysts in the faecal mass were examined, not by sowing some of the faecal mass itself on L.E.S. medium, but by preparing from a small portion of it a washed concentrated suspension of cysts and sowing a few drops of this as described above : this procedure was, for obvious reasons, found to be a more sensitive test of the presence of live cysts than the direct sowing of the faecal material.

Experiments of this nature were performed on a number of occasions, and with infected faeces from several different individuals ; in all cases the results were very similar.

It will be observed from Table I, which sets forth the results obtained in one experiment of this nature, that in each case the ice-chest specimen survives the longer ; and again that the longevity of the cysts is greater in the washed suspension than in the original faecal mass. The maximum period for which we have found cysts to remain viable, at room temperatures, in the faecal mass has never exceeded nine days, and in washed suspensions, ten days ; whereas a saline or water suspension maintained at 0° C. has been found to contain a few viable cysts up to the seventeenth day. It should be noted that in the later periods the cultures were not nearly as luxuriant as on the first few days, and eventually prolonged search was necessary to determine whether they were actually positive or negative.

TABLE I.

Showing the result of culturing *E. histolytica* cysts after storing *in vitro* under various conditions for varying periods of time.

Time in days	Presence or absence of vegetative *Entamoebae* in 16 hour-old cultures			
	Infected faeces stored at 0° C.	Infected faeces stored at 16 to 20° C.	Washed concentrated suspension of cysts stored at 0° C.	Washed concentrated suspension of cysts stored at 16 to 20° C.
1	+	+	+	+
4	+	+	+	+
7	+	+	+	+
9	+	−	+	+
10	−	−	+	+
11	−	−	+	−
15	−	−	+	−
17	−	−	+	−
21	−	−	−	−

RESISTANCE OF *E. HISTOLYTICA* CYSTS TO HEAT

Apparently the only observations on this subject are those of Boeck (1921a), who, using dilute eosin as a test of viability, found that the thermal death point of *E. histolytica* cysts was 68° C.

Our investigations on the point were conducted as follows :—

A washed concentrated suspension of *E. histolytica* cysts was prepared by the sugar method (*vide* previous paper) and two drops of the centrifuged deposit containing very numerous cysts (50 or more to the microscope field, A obj. No. 4 oc.) were added to a series of centrifuge tubes each containing 5 c.c. of water. The tubes were then heated in water baths at various temperatures for 5 minutes and 30 minutes respectively. After the lapse of these periods the tubes were centrifuged, the deposit sown on L.E.S. medium, and the cultures examined for vegetative *Entamoebae* after incubation for 16 to 20 hours.

The results are given in Table II from which it will be seen that, for the periods in question, *E. histolytica* cysts withstand a temperature of 45° C., but are all killed by a temperature of 50° C.

TABLE II.

Showing the result of culturing *E. bistolytica* cysts, which had been exposed to various temperatures for 5 minutes and 30 minutes respectviely.

Temperature to which cysts were exposed	Presence or absence of vegetative *Entamoebae* in 16 hour-old cultures.	
	Length of time for which cysts were exposed to various temperatures	
	5 minutes	30 minutes
40° C.	++	++
45° C.	++	++
50° C.	–	–
55° C.	–	–
58° C.	–	–
60° C.	–	–

THE RESISTANCE OF *E. HISTOLYTICA* CYSTS TO VARIOUS DRUGS AND CHEMICALS

Kuenen and Swellengrebel, by means of the dilute eosin test for viability, found that a solution of sublimate (1 in 1,000) killed *E. histolytica* cysts in four hours, creolin (1 in 250) killed them in five to ten minutes, and 50 per cent. alcohol or Schaudinn's sublimate alcohol solution killed them immediately; on the contrary it is stated that the cysts were very resistant to emetin hydrochloride, and that they survived exposure to a 10 per cent. solution of formalin for a few minutes.

Wenyon and O'Connor, also relying on eosin as a test for viability, found that acid sodium sulphate and chlorinated lime tabloids (B. W. & Co.) as used for water sterilization, had no action on *E. histolytica* cysts; that they withstood 1 in 200 emetin hydrochloride for nine hours; but were killed immediately by cresol (1 in 20), in one minute by a dilution of 1 in 30, and in half-an-hour by 1 in 100; carbolic acid (1 in 40) killed them in fifteen minutes, and in a dilution of 1 in 100 in seven hours: cysts exposed to 1 per cent. formalin for four hours did not stain with eosin, but were much shrunken and distorted, and had every appearance of being dead.

Boeck and Stiles (1923), likewise basing their observations on the reliability of the eosin test as a criterion of viability, make the

astonishing statement that ' The cysts of *E. histolytica* appear to be viable as long as five days in 5 per cent. formalin.'

In order to re-investigate this subject we conducted experiments of the following nature :—

A washed concentrated suspension of *E. histolytica* cysts was prepared by the sugar method, and two drops of the centrifuged deposit added to 5 c.c. of the fluid to be tested, and allowed to remain there for 30 minutes at either laboratory temperature (20° to 25°C.) or at 37°C. The cysts were then precipitated by means of the centrifuge, and after removal of the supernatant fluid washed four times in water to get rid of all trace of the chemical, and then sown on L.E.S. medium, which subsequently was examined for active *Entamoebae* after 16 to 20 hours incubation.

Such a method of testing the effect of drugs and chemicals on *E. histolytica* cysts is, of course, far more drastic than merely adding the disinfectant to the infected faeces, as although the couple of drops of washed concentrated suspension contained large numbers of cysts, yet the other organic matter present was relatively very small in amount, and the total quantity used was practically insignificant as compared with the relatively enormous volume of the disinfectant. The action of a considerable number of drugs and chemicals, in various strengths, was tested on several occasions with the results shown in Table III. It will be seen that although *E. histolytica* cysts are remarkably resistant to such substances as emetin hydrochloride, ' Yatren,' and hydrochloric acid, they are nevertheless fairly sensitive to such common disinfectants as mercuric chloride, formaldehyde, carbolic acid, and lysol. The result of testing the effect of chlorine (and hypochlorous acid) is interesting and important in that although a dilution of 1 in 64 of a saturated aqueous solution of chlorine (representing about ·01 per cent. of chlorine) is sufficient to destroy all the cysts, yet this concentration is infinitely greater than that used to sterilize water of bacteria. The ordinary procedure of adding chlorinated lime to water in quantities known to be sufficient to destroy all *Bacillus coli* is without effect on *E. histolytica* cysts, and in fact the addition of this substance to water contaminated with *E. histolytica* cysts is, for practical purposes, completely useless.

TABLE III.

Showing the result of culturing *E. histolytica* cysts which had been exposed to various chemicals for 30 minutes at laboratory temperature and at 37° C. respectively.

Chemicals to which cysts were exposed	Concentration	Presence or absence of vegetative *Entamoebae* in 16 hour-old cultures	
		Temperature at which cysts were exposed to chemicals	
		Lab. temperature (20 to 25° C.)	37° C.
H Cl	0·2 per cent.	++	++
	1·0 ,, ...	++	+
	5·0 ,, ...	++	−
	7·5 ,, ...	−	−
	10·0 ,, ...	−	−
NaOH	0·2 per cent.	++	++
	1·0 ,, ...	+	+
	2·5 ,, ...	−	−
	5·0 ,, ...	−	−
Cl	*Sat. sol. in water ...	−	−
	1/2 ,, ,, ...	−	−
	1/4 ,, ,, ...	−	−
	1/8 ,, ,, ...	−	−
	1/16 ,, ,, ...	−	−
	1/64 ,, ,, ...	−	−
	1/320 ,, ,, ...	+	−
Chlorinated lime tabloid (B. W. & Co.)	†1 tablet in 65 c.c. water ...	+	−
	1 ,, 325 c.c. ,, ...	++	+
	1 ,, 650 c.c. ,, ...	++	++
	1 ,, 6500 c.c. ,, ...	++	++
Milton‡	100·0 per cent.	−	−
	10·0 ,, ...	−	−
	5·0 ,, ...	−	−
	2·5 ,, ...	−	−
	1·0 ,, ...	+−	+−
	0·5 ,, ...	+−	+−
$HgCl_2$	1 in 500	−	...
	1 in 2,500	−	...
	1 in 12,500	+	...
	1 in 100,000	++	...
Pot. permangnate	1·0 per cent.	+	+
	0·2 ,, ...	++	++
	0·02 ,, ...	++	++
Formaldehyde ...	2·5 per cent.	−	−
	0·5 ,, ...	−	−
	0·2 ,, ...	++	+
	0·1 ,, ...	++	++
	0·05 ,, ...	++	++
Carbolic acid ...	2·5 per cent.	−	−
	1·0 ,, ...	−	−
	0·5 ,, ...	++	++
	0·1 ,, ...	++	++

TABLE III—*contd.*

Showing the result of culturing *E. histolytica* cysts which had been exposed to various chemicals for 30 minutes at laboratory temperature and at 37° C. respectively.

Chemicals to which cysts were exposed	Concentration	Presence or absence of vegetative *Entamoebae* in 16 hour-old cultures	
		Temperature at which cysts were exposed to chemicals	
		Lab. temperature (20 to 25° C.)	37° C.
Lysol	2·5 per cent.	−	−
	1·0 „	−	−
	0·5 „	+	+−
	0·1 „	++	++
Yatren	5·0 per cent.	+	+
	2·5 „	++	++
	1·0 „	++	++
	0·1 „	++	++
Emetin H Cl ...	5·0 per cent.	++	++
	2·5 „	++	++
	1·0 „	++	++
	0·5 „	++	++

++ Signifies Numerous *Entamoebae.*
\+ „ Scanty *Entamoebae.*
+− „ *Entamoebae* present on some occasions and absent on others.
− „ Consistently negative.

* A saturated solution of chlorine in water contains at 24° C. approximately 0·7 per cent. of chlorine by weight.

† A tabloid (B. W. & Co.) of chlorinated lime is equivalent to 0·065 gm. of chlorine.

‡ Stated to contain sodium hypochlorite with a relatively high percentage of hypochlorous acid; the available chlorine is 1·05 per cent.

SUMMARY

1. The work described in this paper indicates that the eosin reaction is not an entirely reliable criterion of viability in so far as *E. histolytica* cysts are concerned; whilst those cysts which stain with dilute eosin are almost certainly dead, it is by no means true that all those which do not stain are alive.

2. A more reliable and satisfactory method of determining the viability of *E. histolytica* cysts, based on the fact that they can be readily cultured *in vitro*, is described; and the following information regarding their longevity, thermal death point, and resistance to chemicals and drugs, has been obtained.

3. *E. histolytica* cysts commence to die fairly rapidly in faeces which has been kept at laboratory temperature (16 to 20° C.) for 3 or 4 days, and all are dead within about 10 days ; approximately the same result is obtained when the faeces is kept at 0° C. in the ice-chest.

4. Washed suspensions of *E. histolytica* cysts in water live rather longer, more especially when kept at 0° C. : but even under these conditions live cysts are not found after three weeks.

5. *E. histolytica* cysts survive a temperature of 45° C. for 30 minutes, but are killed within five minutes by a temperature of 50° C.

6. *E. histolytica* cysts are remarkably resistant to emetin and to ' Yatren ' ; and relatively so to hydrochloride acid and chlorine, the last-named, in strengths far in excess of that used in the bacteriological sterilization of water, having no effect on the cysts. The lethal strengths of solutions of such substances as mercuric chloride, potassium permanganate, formaldehyde, lysol, carbolic acid, and the proprietory preparation ' Milton ' have been ascertained.

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